Protein Kinases in the Lactating Mammary Gland

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ABSTRACT

Protein kinases in the cytosol of whole bovine lactating mammary gland were separated by phosphocellulose chromatography. Five protein kinases (identified as histone kinase and casein kinases A, B, C, and D) were characterized and compared with kinases from other tissues. The histone kinase activity was identified as cyclic AMP-dependent protein kinase. The casein kinases differed in their activities toward a variety of proteins (α_{S1}-casein, native and dephosphorylated β-casein, and α-lactalbumin) and peptides. Based on substrate specificity studies and the inhibitory effects of heparin, 2,3-diphosphoglycerate, and guanosine triphosphate, kinases B, C, and D were tentatively identified as glycogen synthase kinase-3, casein kinase I, and casein kinase II, respectively.

INTRODUCTION

Phosphorylation of proteins and enzymes, an important mechanism in the regulation of cellular activities, is mediated through protein kinases (12, 15, 22). In the lactating mammary gland, these enzymes are distinct from the specific casein kinases responsible for the phosphorylation of milk proteins (caseins). The kinase involved in the phosphorylation of caseins is located in the Golgi membranes and phosphorylates dephosphorylated α_{S1}-, β-, and κ-caseins but not the native caseins (2, 4, 20, 27). Neither cyclic AMP nor calmodulin affect the activity of this enzyme (2). Brooks and Landt (6) described a Ca^{2+} and calmodulin-dependent protein kinase in a microsomal fraction prepared from rat mammary acini which specifically modifies dephosphorylated κ-casein Native κ-casein and α_{S1}-casein (native and dephosphorylated) were not phosphorylated.

Recent studies have examined protein kinases that regulate cellular activities in the mammary gland. Olins and Bremel (19) have shown that physiological concentrations of oxytocin stimulate a rapid phosphorylation of myosin in rat mammary myoepithelial cells. Their results suggest that calcium ions and calmodulin play an important role in the regulation of myosin phosphorylation, which in turn regulates milk ejection.

Munday and Hardie (18) partially purified three cyclic AMP-independent acetyl coenzyme A (CoA) carboxylase kinases from the cytosol of mammary glands of lactating rats. All three enzymes phosphorylate casein and two are similar to casein kinases I and II, enzymes that have been characterized in a number of other tissues (7, 8, 9, 13). These latter two kinases phosphorylate acetyl CoA carboxylase but have no effect on its activity. The third kinase, acetyl CoA carboxylase kinase-2, inactivates acetyl CoA carboxylase through phosphorylation but does not seem to be related to other protein kinases.

This paper describes preliminary experiments on the protein kinase content of the cytosolic fraction from the lactating mammary gland of the cow. Protein kinases were separated by phosphocellulose chromatography and characterized. The ultimate objective is to examine the role of the various protein kinases in the onset and maintenance of lactation.

MATERIALS AND METHODS

Materials

α_{S1}-Casein B (25), β-casein A (10), and κ-casein B (28) were purified from milk of individual cows homozygous for the particular genetic variant. Dephosphorylated β-casein A was prepared by the procedure of Bingham et al. (3). α-Lactalbumin and histone (Type II AS)

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were obtained from Sigma Chemical Company, St. Louis, MO,[2] [γ-^{32}P]ATP was from New England Nuclear, (Boston, MA) and phosphocellulose P-11 was from Whatman (Clifton, NJ).

Peptide 1, a phosphopeptide, was isolated from a tryptic digest of β-casein (16). Peptides 2 and 3 were kindly provided by Bruce Kemp, University of Melbourne, Australia.

Source of Mammary Glands

Bovine mammary glands were obtained from a lactating cow at the Beltsville Agricultural Research Center through the cooperation of John Keys. Following slaughter, mammary glands were trimmed of extraneous fat, cut into pieces (approximately 200 g), frozen, and stored at −20°C until needed.

Protein Kinase Assays

Histone kinase was measured at pH 6.5 in a 100-μl reaction mixture containing 15 m*M* K_2HPO_4/KH_2PO_4, 5 m*M* Mg acetate, 1 m*M* ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), .36 m*M* 3-isobutyl-1-methyl xanthine (phosphodiesterase inhibitor), 40 m*M* NaF, .3 m*M* [γ-^{32}P] ATP (50 to 150 cpm/pmol), 4 mg/ml histone, and, when indicated, 1.5 μ*M* cyclic AMP.

Casein kinase activity was determined at pH 7.0 in a 100-μl reaction mixture containing 50 m*M* Tris-HCl, 140 m*M* KCl, 5 m*M* Mg acetate, 1 m*M* EGTA, 2 mg/ml β-casein A, .14 m*M* [γ-^{32}P]ATP (50 to 150 cpm/pmol), and casein kinase. Where indicated, other proteins and peptides were substituted for β-casein.

The phosphorylation reactions were initiated by the addition of [γ-^{32}P]ATP in 20 μl and were incubated for 20 min at 30°C. The products of the reaction were measured by several methods. The samples from the phosphocellulose columns were analyzed by applying 50 μl of the incubation mixture onto squares (2 × 2 cm) of Whatman No. 31ET chromatography paper. The paper squares were then treated by the method of Reimann et al. (21). In the other experiments, the phosphate incorporated into proteins was measured by the chromatographic method of DePaoli-Roach et al. (8).

When peptides (.5 mg/ml) were used as substrates, the reactions were terminated by the addition of 100 μl of 60% acetic acid. The phosphorylated peptides were then determined by the procedure described by Kemp et al. (14).

Preparation of Protein Kinases

The procedure was based on the method of DePaoli-Roach et al. (8). All operations were carried out at 4°C. Mammary tissue (50 g) was cut into small pieces and homogenized in a Waring Blendor for 1 min in 150 ml buffer (pH 7.5) containing 50 m*M* Tris-HCl, .01 *M* dithiothreitol, 50 m*M* NaF, and .25 m*M* phenylmethylsulfonyl fluoride (PMSF). The crude extract was filtered through cheesecloth and centrifuged at 13,000 × *g* for 40 min. The resulting supernatant was filtered through cheesecloth and centrifuged for 3 h at 70,000 × *g*. The precipitate was discarded. Solid ammonium sulfate was added slowly to the supernatant until 30% saturation was obtained. After stirring for 30 min, the mixture was centrifuged for 30 min at 10,000 × *g*. The supernatant was adjusted to 60% saturation with ammonium sulfate, stirred for 30 min, and centrifuged at 10,000 × *g* for 30 min. The precipitate was dissolved in buffer A, which contained 50 m*M* Tris-HCl, 1 m*M* dithiothreitol, 5% glycerol, and .25 m*M* PMSF (pH 7.5). The solution was dialyzed for 20 h against 4 L of buffer A with two changes, Any insoluble material was removed by centrifugation at 10,000 × *g* for 20 min.

Phosphocellulose Column Chromatography

The dialyzed preparation was applied to a phosphocellulose column, 2 × 25 cm. The column was washed with buffer A until the 280 nm readings of the eluate reached a baseline value and was then washed with 50 ml of buffer A, containing .4 *M* KCl. A gradient (200 ml) formed from equal volumes of buffer A plus .4 *M* KCl and buffer A plus 1.4 *M* KCl was applied. Fractions (3.6 ml) were collected.

RESULTS

Phosphocellulose Chromatography

Protein kinase activities were monitored by the incorporation of radioactive phosphorus into casein and histone (Figure 1). Stimulation of histone kinase by cyclic AMP was considered

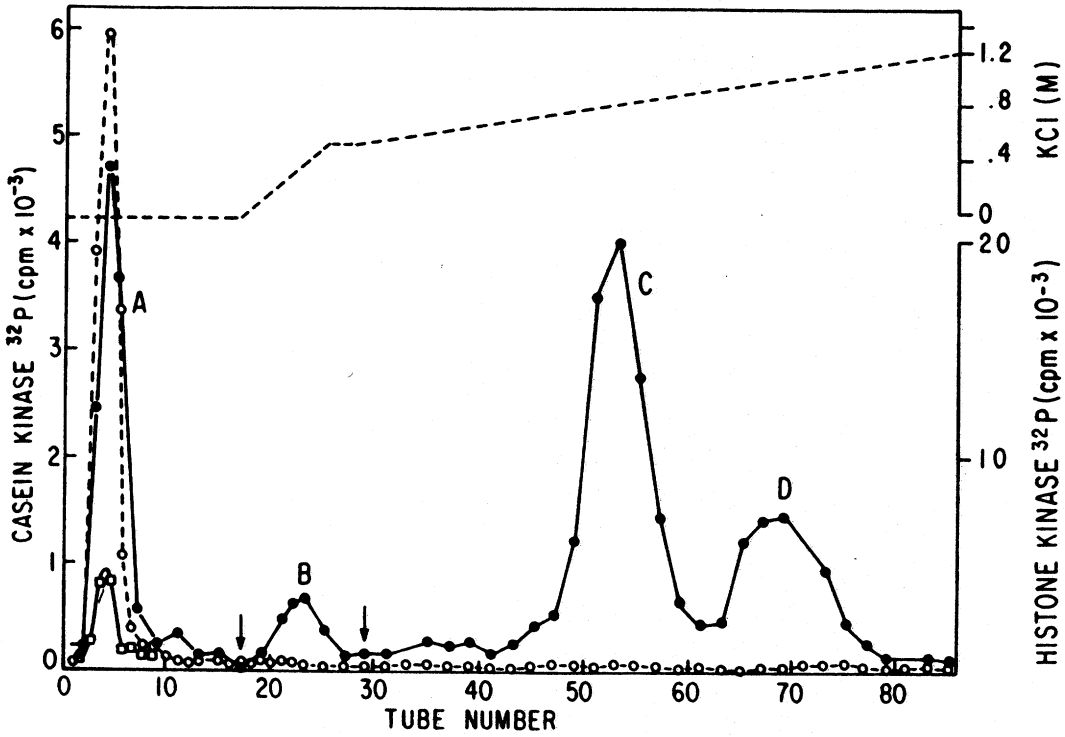

Figure 1. Chromatography of protein kinases on phosphocellulose. Supernatants derived from bovine mammary tissue were analyzed. The first arrow represents elution with .4 *M* KCl and the second arrow indicates start of KCl gradient. Column fractions were assayed by using β-casein A (•) and histone with (○) and without cyclic AMP (□).

to be a measure of cyclic AMP-dependent protein kinase activity. β-Casein A was used to monitor casein kinase activity since this genetic variant is not a substrate for cyclic AMP-dependent protein kinases (5, 14), but is an excellent substrate for casein kinases (26).

Four peaks of casein kinase activity (labeled A, B, C, and D) from the mammary supernatant are shown in the chromatogram (Figure 1). Peak A, which did not bind to the phosphocellulose, contained all the histone kinase activity, most of the protein (data not shown), and a portion of the casein kinase activity. The histone kinase activity was stimulated by cyclic AMP. A minor peak (B) of casein kinase activity was eluted by .4 *M* KCl. Two peaks (C and D) were obtained by a gradient, eluting at .8 *M* and 1.0 *M* KCl, respectively.

Effect of Inhibitors

The effects of various substances on the phosphorylation of β-casein are shown in Figure 2. Heparin, which inhibits casein kinase II (11), was examined as to its effect on the column fractions. Of the four casein kinases, only casein kinase D was significantly inhibited by heparin. Whether guanosine triphosphate (GTP) as well as ATP could serve as a phosphate donor was tested on the four casein kinases. Unlabeled GTP (1 to 2 m*M*) added to .3 m*M* [γ^{-32}P] ATP reduced the activities of casein kinases A, B, and D to less than 10%. However, most casein kinase C remained active in the presence of 2 m*M* GTP, indicating that GTP was not utilized as a phosphate donor by the enzyme. 2,3-Diphosphoglycerate inhibited all the bovine casein kinases but to different extents. In the presence of 8 m*M* 2,3-diphosphoglycerate, the activities of casein kinases A, B, C, and D were 52, 45, 16, and 2%, respectively, of the control.

Substrate Specificity

The four bovine casein kinases showed differences in the rates of phosphorylation of

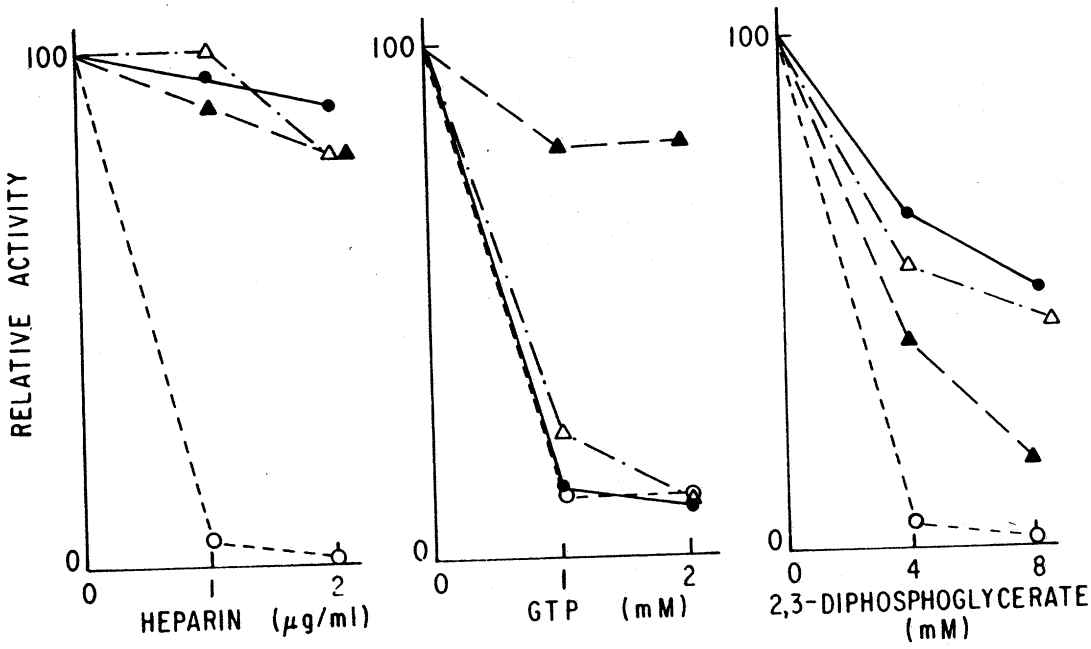

Figure 2. Effect of inhibitors on the activity of cow casein kinases, peak A (●), peak B (△), peak C (▲), and peak (D (○). Casein kinases were assayed under standard conditions except that the indicated concentrations of heparin, guanosine triphosphate (GTP), and 2,3-diphosphoglycerate were added.

various milk proteins (Table 1). Because identification of the four enzyme activities (Figure 1) was based on their ability to phosphorylate β-casein A, this substrate was used to compare the rate of phosphorylation to that of other substrates. Casein kinases A and B phosphorylated α_{s1}-casein, native and dephosphorylated β-casein, κ-casein, and reduced and alkylated (RCM)-α-lactalbumin. The best substrate for casein kinase A was β-casein A, whereas κ-casein was the best substrate for casein kinase B. Casein kinase C phosphorylated α_{s1}-casein and κ-casein in addition to β-casein A but had little activity with dephosphorylated

TABLE 1. Substrate specificity of protein kinases from bovine mammary gland. Rate of phosphorylation was determined using standard assay conditions. Protein substrates were 2 mg/ml.

Substrate	Activity of casein kinase fractions				Relative activity			
	A	B	C	D	A	B	C	D
	(pmol·min^{-1}·ml^{-1})							
α_{s1}-Casein B	775	69	344	23	58	65	56	2
β-Casein A	1330	105	598	993	100	100	100	100
Dephosphorylated β-casein A	624	93	13	43	47	88	2	4
κ-Casein	828	170	264	0	62	161	44	0
RCM-α-Lactalbumin[1]	510	67	32	7	38	63	5	1

[1] RCM-α-lactalbumin is α-lactalbumin reduced and alkylated by the method of Shechter et al. (24).

TABLE 2. Peptide substrates.

1.	Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser(P)-Leu-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ser-Ile-Thr-Arg
2.	Val-Glu-Ser-Leu-Ser-Ser-Ser-Glu-Glu-Ser-Ile-Thr-Arg
3.	Ac-Ser-Asp-Glu-Glu-Val-Glu-His

β-casein and RCM-α-lactalbumin. Casein kinase D is unique because β-casein A was the only substrate that was appreciably phosphorylated.

The bovine casein kinases were assayed for activity with the peptide substrates listed in Table 2. Peptide 1, which represents the N-terminal region of β-casein, contains serine 22, threonine 24, and phosphoserines 15, 17, 18, and 19. Peptide 2 is a synthetic peptide containing residues 13 to 25 of peptide 1 and contains five serines and one threonine, none of which are phosphorylated. Peptide 3 is a synthetic peptide identical to the N-terminal region of troponin T and has one serine residue. The rates of phosphorylation of the three peptides were compared to the rate of phosphorylation of β-casein A (Table 3). Casein kinase A phosphorylated the three peptides at rates similar to that of β-casein A; peptide 2 was the best substrate; the rate was 1.5 times that of β-casein A. Casein kinase B phosphorylated all the peptides at rates which were 20 to 30-fold higher than those of β-casein A. Casein kinases C and D phosphorylated the peptides at relatively low rates. However, a small degree of phosphorylation of peptide 3 was observed with casein kinase D. Each of the four casein kinases phosphorylates peptides 1 and 2 at similar rates. Because the major difference between the two peptides is the endogenous phosphate in peptide 1, the presence of phosphate groups in the four serine residues in peptide 1 appeared to have little effect on the rate of phosphorylation.

DISCUSSION

Phosphocellulose chromatography provides a tool for separating the protein kinases of mammary gland cytosol. Five protein kinases were identified in the lactating mammary tissue of cow. One protein kinase can be classified as cyclic AMP-dependent protein kinase by three criteria. This kinase modifies histone, is stimulated by cyclic AMP, and does not bind to phosphocellulose, a property also observed by other investigators (8, 13).

Table 4 compares mammary casein kinases to other well-characterized casein kinases. In analyzing these data, it was assumed that inhibition by GTP is due to the ability of casein kinase to use GTP as well as ATP as a substrate.

TABLE 3. Phosphorylation of peptide substrates by bovine casein kinases. Rate of phosphorylation was determined using standard assay conditions with peptide concentrations of .5 mg/ml. Concentration of β-casein was 2 mg/ml. Specific peptides are identified in Table 2.

Substrate	Activity of casein kinase fractions				Relative activity			
	A	B	C	D	A	B	C	D
	(pmol·min^{-1}·ml^{-1})							
Peptide 1	1100	2980	10	0	94	2865	2	0
Peptide 2	1730	3160	0	20	148	3038	0	2
Peptide 3	1260	2110	20	150	108	2028	4	18
β-Casein A	1172	104	492	847	100	100	100	100

TABLE 4. Comparison of mammary casein kinases with other casein kinases. Activities are expressed as percentages with activity toward β-casein equal to 100.

Property	A	B	F_A/GSK-3[1]	C	Casein kinase 1[1]	Casein kinase II (PC .7)[1]	D	Casein kinase II (PC.7)[1]	Casein kinase II[2]
Substrate									
α_{S1}-Casein B	55	65	42	56	12	35	2	5	5
β-Casein A	100	100	100	100	100	100	100	100	100
β-Casein (dephosphorylated)	47	88		2		6	4		17
κ-Casein	62	161	167	44	38		1	6	
Inhibitors									
Heparin	–	–		–	+	–	+	+	+
GTP[3]	+	+		–			+		
Uses GTP[3]			+		–	–		+++	+++

[1] From Ahmad et al. (1). F_A/GSK-3 = Clycogen synthase kinase-3.

[2] From Tuazon et al. (26).

[3] Guanosine triphosphate.

When the phosphate from GTP is transferred to protein, labeled phosphate from ATP is diluted.

Casein kinase A elutes from the phosphocellulose column at the front with cyclic AMP-dependent protein kinase. Therefore, some of the properties of casein kinase A could be attributed to cyclic AMP-dependent protein kinase. For example, both enzymes phosphorylate κ-casein (5). However, there are characteristics that distinguish casein kinase A from cyclic AMP-dependent protein kinase. β-Casein A and α_{s1}-casein, poor substrates for cyclic AMP-dependent protein kinase, were phosphorylated by casein kinase A (5). In addition, GTP, which has no effect on cyclic AMP-dependent protein kinase (22), inhibited the reaction. More detailed studies will require purification and separation of the two enzymes.

Casein kinase B can be compared with F_A/GSK-3 (also called F_A or glycogen synthase kinase-3). κ-Casein is the preferred substrate for both kinases. The two enzymes can also modify other caseins, are not inhibited by heparin, and can use GTP as a phosphate donor. However, identification of casein kinase B with F_A/GSK-3 should be regarded as tentative pending further studies.

Casein kinase C resembles casein kinase-1 (1) and casein kinase I (26). Caseins (α_{s1}, β, and κ) are good substrates, but dephosphorylated β-casein is not appreciably phosphorylated. Guanosine triphosphate has little or no effect on the phosphorylation. Casein kinase C is not appreciably inhibited by heparin and, in this respect, is similar to casein kinase I (11) but differs from casein kinase-1, which is inhibited by heparin. Generally heparin has little effect on casein kinase I-type enzymes. However, some investigators have found that heparin inhibits this enzyme (1, 18). The reason for this discrepancy is not clear. Casein kinase C was inhibited by 2,3-diphosphoglycerate, but the inhibition was considerably less than that observed with casein kinase D (Figure 2). Hathaway et al. (12) reported similar results; the I_{50} value for casein kinase I was sevenfold higher than that of casein kinase II.

Casein kinase D, PC .7, and casein kinase II have similar properties. β-Casein A is phosphorylated to a much greater extent than α_{s1}-casein, κ-casein, or dephosphorylated β-casein. The three enzymes can use GTP and are inhibited by heparin.

The four casein kinases show significant differences when the peptide substrates are tested (Table 3). Phosphorylation of β-casein by kinase B is considerably reduced over that observed with the shorter peptides. Either β-casein inhibits phosphorylation or the site of phosphorylation is not as accessible to the enzyme. Tuazon et al. (26) showed that serine 22 in β-casein is the site that is phosphorylated by casein kinase I. However, very little phosphorylation of the short peptide containing this serine residue occurs. Peptides 1 and 2 do not contain threonine 41, the residue that was phosphorylated by casein kinase II (26); therefore, these peptides would not be expected to be substrates for kinase D. However, peptide 3, which contains a serine residue followed by three acidic amino acids, can be phosphorylated. A similar peptide (AcSer-Glu-Glu-Glu-Val-Glu) was phosphorylated by rat liver casein kinase II, but not by casein kinase I (17). Whereas the four casein kinases showed little differences in the rate of phosphorylation of peptides 1 and 2, the casein kinase associated with the Golgi membranes exhibited a strong preference for peptide 1 after the phosphate groups had been removed (4).

These studies provide a preliminary characterization of the cyclic AMP-dependent and independent protein kinases of the bovine lactating mammary gland. The cytosolic fraction analyzed in this study represents a combined soluble fraction from a variety of cell types, which include lactating epithelial cells, myoepithelial cells, endothelial cells, ductal cells and fibroblasts. Because the mammary tissue used in this study was from a midlactation animal of average production, it is expected that lactating cells would predominate (23). Experiments are in progress to isolate and further characterize these kinases and to determine whether these kinases are associated with the general metabolism of the gland or whether they are induced during pregnancy and lactation.

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